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Effects of Two Antibiotic Regimens on Course and Persistence of Experimental *Chlamydia pneumoniae* TWAR Pneumonitis

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We studied the effects of two antibiotic regimens on the course of *Chlamydia pneumoniae* infection in the lungs of Swiss Webster mice. After intranasal challenge with isolates AR-388 (1.3×10^7 inclusion-forming units per mouse) and AR-39 (1.5×10^6 inclusion-forming units per mouse), groups of animals were treated with either doxycycline (10 mg/kg of body weight once a day for 3 days), azithromycin (10 mg/kg [single dose]), or saline. Responses were assessed by the isolation of organisms in cell culture, detection of TWAR DNA in lung tissues by PCR, and lung histology. Both regimens were effective in clearing infections induced by AR-388 ($P = 0.02$ and 0.007 for doxycycline and azithromycin, respectively) compared with controls. TWAR DNA was detected in 77 and 25% of culture-negative lungs 2 weeks after treatment of AR-388 and AR-39 infections, respectively. Histological changes showed interstitial pneumonitis and were similar over time for all groups. Single-dose azithromycin produced drug levels in lung tissues above the MICs for the test strains for a period three times longer than that of single-dose doxycycline. We concluded that short-term antibiotic regimens were successful for the treatment of experimental TWAR pneumonitis in mice. TWAR DNA was frequently recovered from lung tissues after apparently successful treatment.

Chlamydia pneumoniae (TWAR) is a frequent cause of community-acquired pneumonia in adults, accounting for 6 to 12% of cases (14, 29). Clinical and radiographic features are not specific, and laboratory diagnosis is necessary (10, 13, 30). Unfortunately, isolation in cell culture systems and serology by the microimmunofluorescence test are cumbersome and laboratory diagnosis is often delayed (14).

Currently, recommendations for treatment are based on in vitro sensitivity studies, analogies to the treatment of other chlamydial infections, and anecdotal reports for humans (9, 13, 17, 44, 45). It has been suggested that prolonged antibiotic therapy is necessary to cure TWAR infections (14, 17, 18, 45). This recommendation followed observations of continuing symptoms, in some cases with repeatedly positive cultures, after primary infection and antibiotic treatment for 10 days. Persistent infections with other chlamydial species after short-term treatment in controlled trials are well established (40). Moreover, the question of *C. pneumoniae* persistence after treatment is potentially of great importance since *C. pneumoniae* has been associated with chronic diseases, such as atherosclerosis and coronary heart disease, in seroepidemiological studies (28, 32, 38, 39, 46, 47) and by direct detection of *C. pneumoniae* in atheromatous lesions (5, 24, 27, 43).

We recently developed an experimental model of TWAR pneumonitis in mice which could be used to address some of these issues (50). In this study, we investigated the effects of two antibiotic regimens, doxycycline administered for 3 days and single-dose azithromycin, on the course of pneumonitis and the persistence of organisms after treatment. The recently demonstrated effectiveness of a single dose of azithromycin for the treatment of chlamydial urethritis and cervicitis takes advantage of sustained high drug concentrations in cells and tissues (31).

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MATERIALS AND METHODS

***C. pneumoniae* strains and inoculum.** TWAR strains AR-388 and AR-39, two pharyngeal isolates, were used in this study (15). Both strains were grown in HL cells (26). Infected cells were harvested with sterile glass beads and ultrasonically disrupted. Cell culture-grown organisms were partially purified by one cycle of low- and high-speed centrifugation each, resuspended in sucrose-phosphate-glutamic acid (SPG) buffer, and frozen in 0.5-ml aliquots at −70°C. Inoculum preparations contained 2.6×10^8 inclusion-forming units (IFU) of AR-388 per ml and 3×10^7 IFU of AR-39 per ml.

Experimental animal model. Four-week-old male Swiss Webster mice were inoculated intranasally with 1.3×10^7 IFU of AR-388 and 1.5×10^6 IFU of AR-39 under light ether anesthesia to induce hyperventilation. Antibiotic treatment was started 2 days after inoculation, when pneumonitis was most severe. Groups of 12 to 15 animals were killed by axillary bleeding at different time points after treatment to assess viable counts of organisms in lung tissue, TWAR DNA in tissue, and histology. In some groups, blood was saved to obtain serum samples for serological assays. At sacrifice, lungs were removed in toto; three-fourths of one lung was further processed for culture, and the other one-fourth was frozen at −70°C for DNA detection. Lungs from separate animals were removed in toto and placed in 10% formalin for histology.

Antibiotic treatment. Two days after inoculation, animals were randomly chosen to be injected intraperitoneally (i.p.) either with phosphate-buffered saline (PBS) once a day for 3 consecutive days, with doxycycline hyclate (DOXY 100; Lymphomed, Deerfield, Ill.) at 10 mg/kg of body weight once a day for 3 consecutive days, or with azithromycin dihydrate (kindly provided by Pfizer Research Laboratories, Groton, Conn.) at 10 mg/kg on day 1 and PBS on days 2 and 3. Doxycycline was dissolved according to the manufacturer's instructions. Azithromycin dihydrate was dissolved in ethanol and further diluted in PBS before injection.

Culture of lung tissue. Lungs were weighed, minced, and homogenized to make a 10% (wt/vol) suspension in cold SPG buffer. Tissue suspensions were centrifuged at 500 × *g* for 10 min at 4°C to remove debris and frozen at −70°C until tested. Viable counts were assayed by titration of tissue homogenates in HL cells grown on a 12-mm-diameter coverslip in a flat-bottomed 1-dram (4-ml) shell vial. Inoculated cells were incubated at 36°C for 4 days; infected cells were fixed with acetone and stained with a *Chlamydia* genus-specific monoclonal antibody (CF-2) conjugated to fluorescein isothiocyanate (23). Inclusions were counted under a fluorescence microscope. Viable counts were expressed as $\log_{10}$ IFU per gram of lung tissue.

Detection of TWAR DNA in lung tissues. Frozen tissues were thawed and homogenized in a DNA extraction buffer containing 50 mM Tris (pH 8), 25 mM EDTA (pH 8), 2% Triton X, and 20 μg of proteinase K per ml. DNA was isolated from tissues with a DNA extraction kit (Qiagen Inc., Chatsworth, Calif.). PCR was done with *C. pneumoniae*-specific HL-1 and HR-1 primer sets, which results in an amplified product of 437 bp (4). Amplified products were visualized by agarose gel electrophoresis and confirmed by Southern hybridization with cloned DNA of the target sequence as a probe. DNA probes were labeled by

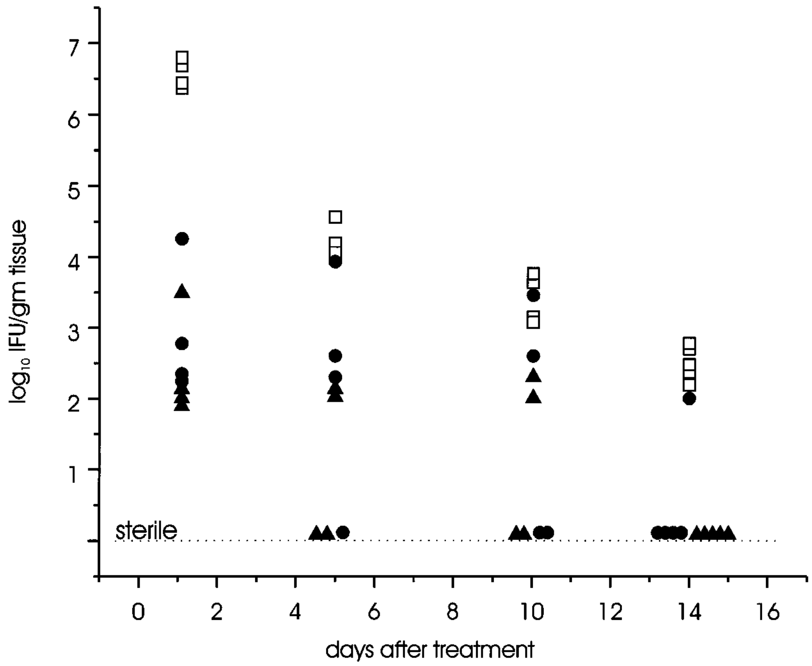

FIG. 1. Recovery of organisms from lungs of mice inoculated with AR-388 (1.3×10^7 IFU per mouse) and treated with PBS (squares), azithromycin (triangles), and doxycycline (circles). Each symbol represents the result of quantitative lung culture from one animal. Groups of animals were sacrificed 1, 5, 10, and 14 days after treatment. sterile, culture-negative lungs.

using the Genius labeling and detection kit (Boehringer Mannheim, Indianapolis, Ind.). Hybrids were detected by immunochemiluminescence with Lumi Phos 530 (Boehringer Mannheim). Extraction controls and tissue controls from uninfected animals were run in parallel. In early experiments, inhibitors of PCR were detected frequently. Inhibitors were removed by drop dialysis (type VS filter, 0.025-μm pore size; Millipore, Bedford, Mass.), and amplification was done with sample volumes of 1 and 5 μl. The data shown are pooled from all experiments.

In vitro susceptibility of *C. pneumoniae* to doxycycline and azithromycin. In vitro drug susceptibility testing was done as previously described, with minor modifications (25). In brief, AR-388 and AR-39 were grown in HL cells at inocula to yield 2 to 5 inclusions per field at a magnification of ×400 in controls. After centrifugation, the inoculum was removed and replaced by culture media containing doxycycline or azithromycin at twofold dilutions. After incubation for 3 days, cells were stained and examined for inclusions under a fluorescence microscope. Unstained cells were harvested, passed, cultured without antibiotics for 3 days, and examined. MICs were defined as concentrations causing the complete inhibition of inclusion formation in the original inoculum; MBCs were defined as concentrations causing the complete inhibition of inclusion formation in passage 2.

Serology. Serum antibody was detected by the microimmunofluorescence test, with formalin-fixed whole elementary bodies of AR-39 as antigen (48). Immunoglobulin G serum antibody fractions were measured by using fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (heavy chain specific; Sigma Chemical Co., St. Louis, Mo.). Antibodies were assayed by using a single dilution of serum samples at 1:8.

Levels of doxycycline and azithromycin in serum samples and lung tissues. Four-week-old male Swiss Webster mice were injected i.p. with a single dose of either doxycycline hyclate (10 mg/kg of body weight) or with azithromycin dihydrate (10 mg/kg of body weight). Groups of three to five animals were sacrificed 3, 8, 18, and 24 h after injection of doxycycline and 2, 6, 12, 24, 48, and 72 h after injection of azithromycin. At each time point, animals were killed by axillary bleeding, serum samples were collected, and lungs were removed in toto and frozen at −70°C. Drug levels in serum samples and lung tissues were determined by high-performance liquid chromatography as previously described (42, 44). Azithromycin levels were kindly measured by G. Foulds, Pfizer Research Laboratories, and doxycycline levels were kindly measured by V. Ainardi, Children's Hospital, Seattle, Wash.

Histopathology. Formalin-fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin by standard methods.

Statistics. The proportion of sterile animals in the course of pneumonitis among the three treatment groups was analyzed by Fisher's exact test.

RESULTS

In vitro susceptibility of *C. pneumoniae* strains. The in vitro drug susceptibilities of AR-388 and AR-39 were identical. Both test strains were susceptible to the inhibitory and bactericidal activities of doxycycline (MIC and MBC, 0.05 μg/ml) and azithromycin (MIC and MBC, 0.1 μg/ml). Similar data have been reported previously (19, 49).

Levels of doxycycline and azithromycin in serum samples and lung tissues from mice. Three hours after a single dose of 10 mg/kg i.p., doxycycline produced peak levels (mean ± standard error of the mean) in serum samples and lung tissues of 1.09 ± 0.08 μg/ml and 0.53 ± 0.21 μg/g, respectively. Levels in serum samples and lung tissues remained above the MICs for the test strains for 18 h (0.13 ± 0.02 μg/ml and 0.09 ± 0.01 μg/g, respectively). After 24 h, drug concentrations in serum samples and tissues were below the lower limits of quantification, 0.04 μg/ml and 0.04 μg/g, respectively. After a single dose of 10 mg/kg i.p., peak concentrations of azithromycin in serum samples were low (0.149 ± 0.043 μg/ml) and in the range of the MICs for the test strains only at 2 h after injection. In contrast, concentrations of the drug in lung tissues were very high at the same time (10.39 ± 1.9 μg/g), and concentrations in tissues remained well above the MICs for the test strains during 48 (0.8 ± 0.5 μg/g) to 72 (0.4 ± 0.16 μg/g) h after injection.

Course of pneumonitis following antibiotic treatment. The results as assessed by quantitative cultures of the organisms in lung tissues from control and antibiotic-treated animals are shown in Fig. 1 and 2.

(i) Inoculation with AR-388 (Fig. 1). Control animals showed a decline in IFU counts per gram of tissue of 4 $\log_{10}$ over 14 days. Up to 14 days after the end of treatment, organisms could still be cultured from all controls, while all animals in the azithromycin group and all but one animal in the doxycycline group were culture negative. Ten days after treatment,

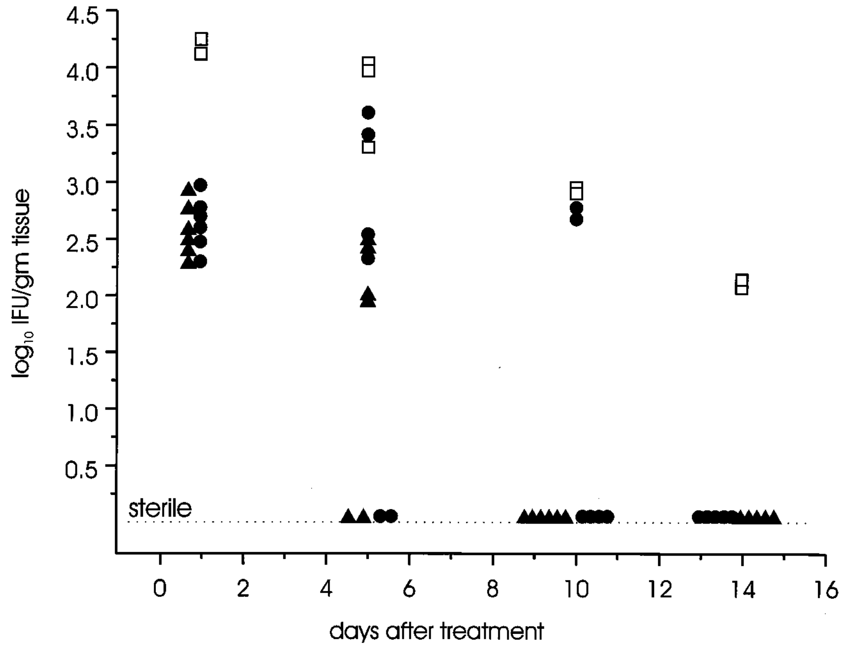

FIG. 2. Recovery of organisms from lungs of mice inoculated with AR-39 (1.5×10^6 IFU per mouse) and treated with PBS (squares), azithromycin (triangles), and doxycycline (circles). Each symbol represents the result of quantitative lung culture from one animal. sterile, culture-negative lungs.

50% (four of eight) of antibiotic-treated animals were culture negative. Both antibiotic regimens cured infections effectively, as assessed by lung cultures, compared with controls ($P = 0.02$ and 0.007 for doxycycline and azithromycin, respectively).

(ii) Inoculation with AR-39 (Fig. 2). Controls showed a decline in IFU counts per gram of tissue of 2 $\log_{10}$ over 14 days. Organisms could still be cultured from all controls 14 days after the end of treatment, while all antibiotic-treated animals were culture negative. Ten days after treatment, 83% (10 of 12) of antibiotic-treated animals were culture negative. Both antibiotic regimens cured infections effectively, as assessed by lung cultures, compared with controls ($P = 0.04$ and 0.03 for doxycycline and azithromycin, respectively).

There was no difference between the two antibiotic regimens with regard to efficacy against infections induced by AR-388 or AR-39.

TWAR DNA in culture-negative lungs of antibiotic-treated mice. The results of the detection of TWAR DNA are shown in Table 1. DNA was detected in 77% (7 of 9) of sterile lungs 14 days after the end of treatment with the higher inoculum of AR-388 and in 25% (3 of 12) of sterile lungs 14 days after the end of treatment with the lower inoculum of AR-39. DNA was never detected in lung tissues from uninfected animals sacrificed in parallel.

TABLE 1. Detection of *C. pneumoniae* DNA by PCR in culture-negative lungs of antibiotic-treated mice

Day after treatment	No. of lungs PCR positive/no. of lungs culture negative[a]			
	AR-388		AR-39	
	Doxycycline	Azithromycin	Doxycycline	Azithromycin
5	1/1	1/2	0/2	1/2
10	1/2	1/2	1/4	4/6
14	3/4	4/5	1/6	2/6

[a] Inocula of AR-388 and AR-39 contained 1.3×10^7 and 1.5×10^6 IFU per mouse, respectively. Treatment regimens consisted of doxycycline at 10 mg/kg of body weight once a day for 3 days and a single dose of 10 mg of azithromycin per kg of body weight.

Serology. Serum antibody was assayed in all animals sacrificed 14 days after the end of treatment. In our previous studies, mice were shown to seroconvert at this time postinfection (50). All animals in the control and treatment groups developed immunoglobulin G antibody against TWAR as demonstrated by the microimmunofluorescence test.

Histopathology. Histological changes over time were similar in severity and extension among the control and treatment groups. After treatment, interstitial pneumonitis was most severe on day 1, with predominantly polymorphonuclear leukocyte infiltration. At days 10 and 14 after therapy, infiltrates were mainly mononuclear cells. At those times, perivascular and peribronchial lymphoid cell accumulations were seen in all groups.

DISCUSSION

Both treatment regimens, doxycycline administered for 3 days and single-dose azithromycin, were similarly effective for the treatment of pneumonitis. Both antibiotics were used at dosages that produced concentrations similar to those achieved in humans after an oral dose of 100 mg of doxycycline and 500 mg of azithromycin, respectively (1, 8, 11, 37). Doxycycline concentrations in serum samples and lung tissues were above the MICs for the test strains for about 18 h. In contrast, azithromycin concentrations in pulmonary tissues were 90 times greater than those in serum samples and persisted above the MICs for the test strains for 48 to 72 h after injection. The importance of the extravascular pharmacokinetics of azithromycin for in vivo efficacy has previously been shown in other experimental models of bacterial infection (12). This might be particularly relevant for the treatment of infections due to intracellular pathogens such as *C. pneumoniae*. Although the 3-day doxycycline regimen may have produced levels in tissues above the MIC for a somewhat shorter period than did single-dose azithromycin, the regimen proved to be similarly effective. We found that a single dose of doxycycline at 10 mg/kg was not effective for the treatment of TWAR pneumonitis in mice (data not shown). Thus, it appears that drug levels above the MIC in lung tissues for several days are required to inactivate TWAR organisms in lungs.

The histopathological changes observed were similar to those previously reported (50). However, the inflammatory lesions were less severe than those in our earlier studies, most likely because of the lower inocula used here. We could not find any appreciable difference over time among the three treatment groups in regard to the extension and severity of histopathological changes. Methodological problems, such as the relatively small sample size studied and difficulties in quantifying the lesions reliably because of irregularly distributed interstitial pneumonitis, could account for this observation. However, similar observations have been made in other experimental systems of chlamydial infection (35). *Chlamydia trachomatis* is well known to produce immunopathological lesions (16, 33, 36). Whether similar mechanisms are operative in TWAR infections needs further study.

On the basis of cell cultures of lung organisms alone, a short course of azithromycin or doxycycline appears to rapidly clear lungs of culturable organisms. However, chlamydiae are known to produce persistent infection in the absence of therapy or after inadequate therapy (3, 18, 22). Careful follow-up studies after treatment are therefore crucial. Follow-up controls based on the culture of organisms alone are problematic for two reasons. First, even if performed under ideal conditions, the sensitivity of culture techniques is limited. Second, after the use of drugs such as azithromycin, with high and sustained levels in cells and tissues, the carryover of the antibiotic may produce false-negative results. Our results show that TWAR DNA can be recovered up to 2 weeks after apparently successful treatment of pneumonitis as assessed by culture. TWAR DNA was recovered more frequently from culture-negative animals that received the higher inoculum of AR-388. While DNA detection as performed cannot distinguish between living and dead organisms, data from other complicated chlamydial infections suggest that the presence of chlamydial DNA might represent the persistence of viable organisms in an unculturable state. Several studies of trachoma in an experimental monkey model and in humans have shown that in the presence of moderate to severe clinical disease, nucleic acid detection at sites of inflammation persists well beyond the detection of organisms by cell culture or fluorescent-antibody techniques (2, 7, 20, 21, 41). A recent study of women with postinfectious tubal infertility detected evidence of *C. trachomatis* by in situ hybridization, immunoperoxidase staining, and electron microscopy in culture-negative tubal biopsy specimens (34). Interestingly, the detection of *C. trachomatis* by methods other than culture was possible even months after antichlamydial treatment. In a monkey pocket model of *C. trachomatis* salpingitis, in situ hybridization detected chlamydial DNA at sites of inflammation up to 4 weeks after inoculation, when culture was negative, while inoculation of animals with UV-inactivated organisms produced no lesions and DNA was not detected (6). Our animal model provides a useful tool to further address these issues in regard to *C. pneumoniae* pneumonitis.

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